Studies in Bacterial Variability.—On the Occurrence and Development of Dys-agglutinable, Eu-agglutinable and Hyper-agglutinable Forms of Certain Bacteria. (A Report to the Medical Research Council.)

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Introductory.

That different cultures of an agglutinable bacterium may exhibit wide differences in relative agglutinability when tested with the same agglutinating serum is a familiar fact. But the conditions on which these differences depend remain to a great extent obscure. Yet a number of facts which bear upon the problem have come to light in the course of investigations carried out by various observers.

A good many years ago the present writer showed (1901) (1) that if a series of strains* of *B. typhosus* be employed in preparing a corresponding series of agglutinating serums, each such serum is found to act more powerfully upon its homologous culture than upon any of the heterologous strains. It was, therefore, stated that the serums were not only specific for the species of bacterium in question, but also *special* in each case to the particular strain employed in its production. It also appeared that, so far as the evidence went, the heterologous strains always fell into the same order of relative agglutinability when tested with the different "special" serums.

Somewhat similar results have recently been published by A. D. Gardner (1920) (2), in connection with his investigation of Paratyphoid A. serums, and he has shown that the peculiar sensitiveness of a given strain to agglutination by serum prepared with that particular strain, which I had indicated by the term "special" is, in part at least, a matter of velocity of reaction. The phenomenon (so far as it depends on reaction rate) was, therefore, spoken of by him as a "super-specific acceleration" of reaction.

Since my own experiments were made before the introduction of the present accurately standardised methods of determining agglutination titre, I had re-examined the question in another relation (1918) (3) with four strains of *B. typhosus.* A rabbit was immunised successively with three of the strains, and on each occasion the titre of its serum rose to a higher level for the strain used as antigen on that occasion, than for either of the other three strains. Furthermore the four strains were seen to belong, two and two, to two serological groups which were widely differentiated from each other by their relative sensitiveness to agglutination by different serums.*

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* In the use of the terms "strains" nothing is implied with regard to the cultures so spoken of save that they have been obtained from different sources, derived by different methods of cultivation, or selected in any way for a particular purpose. If they show differences, it is a matter for investigation in each case, whether such differences are permanent or fleeting.

Somewhat similar serological differences are now well known in certain other organisations, *e.g.*, Meningococcus, Pneumococcus; and they may at times be very marked indeed, so that particular strains are described as "inagglutinable"—for example inagglutinable strains of *B. typhosus.* Yet it is found that on sub-culture these inagglutinable strains will sooner or later yield cultures which give good agglutination. Thus the inagglutinability appears to be only a phase, or temporary character. The inagglutinable form of the organism *cannot, therefore, on the existing evidence be regarded as mutant,* in the proper sense of the term; but it presents an example of bacterial *fluctuation.*

Since the observations just referred to (1918) (3) raised a number of interesting questions the subject has been re-examined in detail during the progress of the present investigation by A. D. Gardner and Ainley Walker (1921) (4) who obtained inagglutinable strains of *B. typhosus,* and compared them serologically with ordinary "good agglutinators." The existence of the two serologically different types of *B. typhosus* was fully confirmed. It was shown that they corresponded to a motile and a non-motile phase respectively of the bacillus. And it was further shown that *from a single culture* of the bacillus colonies could on occasion be isolated by plating which would on cultivation give rise to populations differing as widely, both serologically and as regards motility, as any strains obtained from different sources.

The serological difference between the agglutinable (motile) form and the inagglutinable (non-motile) form of the bacillus was an antigenic difference. For if a serum were prepared with the inagglutinable form, it agglutinated its own bacillus and other inagglutinable strains quite well, and to high titre. But in contrast with the rapidly-formed, large and fluffy flocculi produced in an ordinarily well-agglutinating culture, the clumps produced in inagglutinable suspensions are always small, compact and slowly formed.

On this account, and in view of what is to follow, I propose the term *dys-agglutinable*† as a more appropriate designation than "inagglutinable" for this phase of the bacillus. The ordinarily well-agglutinable phase may then be spoken of as *eu-agglutinable* (where distinction is necessary) and the term *hyper-agglutinable* employed for phases where agglutinability is found to be considerably increased. The differences in agglutinability, and the antigenic differences in strains from different sources, and in sub-cultures from different colonies studied by Gardner and myself arose without experimental interference; that is to say they were naturally occurring differences. So also appear to be the differences recently described in *B. dysenteriæ* (Shiga and Flexner—Y), and several other organisms including *B. typhosus,* in an important paper by Arkwright (1921) (5). Vines also has described and studied strains of meningococcus " hypersensitive " to agglutination (1918) (6) as naturally occurring types. But the results to be recorded show that it is possible to produce experimentally similar differences of agglutinability in many varities of bacteria.

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* A more complete record of this experiment was published by Gardner and Ainley Walker (1921) (4).

† This is a hybrid word; but so are a good number of other accepted and useful terms.

In the course of experiment carried out in 1901–2, I found that the agglutinability of *B. typhosus* was lessened by growing it in a succession of sub-cultures in typhoid agglutinating serum diluted with ordinary culture bouillon (1902) (7). This diminution of agglutinability, and certain other phenomena observed, were interpreted in terms of Erlich's theory, with the conceptions of which I was then imbued, and that interpretation needs reconsideration and may require revision in the light of present knowledge. For it is evident that a diminution in the agglutinability of an organism occurring under these conditions is open to more than one possible explanation, since the production of a relatively inagglutinable race of bacilli might be brought about in several different ways.

Thus it might be due (1) to the gradual training and education of the *whole mass of the population* in successive generations to resist the action of agglutinins; for example by the formation of anti-agglutinins, as I formerly concluded; (2) or it might be due to a selective encouragement of the *propagation of the less agglutinable* individuals (in a population composed of elements differing widely in agglutinability) by some cause facilitating their multiplication in successive generations, to the gradual exclusion and elimination of the more agglutinable individuals; (3) or both processes might play a part.

The first process would offer an example of the heightening and development of a selected character, originally latent, but more or less common to the individuals composing the bacterial population; the second, one of the selective propagation, from a population whose members differed widely among themselves in this respect, of particular individuals in which that character was *already* highly developed. Put briefly, the one would be the *selection of a character* for artificial increase, the other the *selection of individuals* for multiplication. And it is obvious that both processes might be at work side by side.

In view of these considerations, it seemed likely that information of interest would be obtained by inquiring what degree of parallelism, if any, existed between the differences in agglutinability to be observed in different strains (however derived), and those obtainable by repeated cultivation in agglutinating serum. In particular it was hoped to throw some light on the nature and origin of so-called "serological strains" in different species of bacteria. Accordingly experiments have been carried out as described below. Meanwhile the experiments of Gardner and myself already referred to (*loc. cit.*) have shown that extremely wide differences in agglutinability, such as might well form a basis for either process of selection, do actually exist between the individuals which constitute the population of a single culture.

In the account which follows, the experimental work has been summarised so far as possible, and detailed data are recorded only for *B. typhosus*, in order to save space. A general survey of the phenomena under investigation is given, but only a selection of the experiments is recorded in illustration. In certain directions the results are preliminary, and a number of points remain to be worked out in detail.

The Experimental Production of Dys-agglutinable and Hyper-agglutinable Strains (or Phases) of Bacteria.

By repeated subculture in specific agglutinating serums diluted appropriately with culture bouillon, *dys-agglutinable* forms have been obtained of the following bacteria, ten in all, which were all taken from strains then in use in the department: *B. typhosus*, *B. paratyphosus A.*, *B. paratyphosus B.*, *B. ærtrycke*, *B. enteritidis* (Gaertner), *B. coli*, *B. dysenteriæ* (Shiga), *B. dysenteriæ* (Flexner), V, W and Z.

On transference from the serum bouillon to ordinary culture bouillon, these dys-agglutinable forms yield cultures which agglutinate with difficulty, if at all, with corresponding ordinary agglutinating serums; and those of them that were originally motile organisms are found to have passed into a very feebly motile or entirely non-motile phase. After formolisation and dilution to the opacity of standardised agglutinable cultures, they yielded good and uniform suspensions for agglutination tests, though in a number of cases this result was not obtained immediately or very easily. The dys-agglutinable phase of *B. typhosus* and of *B. dysenteriæ* (Flexner), V, W and Z readily yielded excellent suspensions on a number of occasions.

In other cases difficulty sometimes occurred for one of two reasons—

firstly, that in the case of the motile bacilli, the non-motile phase, when first obtained, often shows a tendency to revert very quickly to the motile eu-agglutinable phase in bouillon culture; and, secondly, because, when rapid reversion does not occur, organisms in this group, and also in the dysentery group, which have been grown in a succession of cultures in dilutions of the homologous agglutinating serum, most often grow in early bouillon subcultures in the form of a deposit with clear supernatant fluid. Although these deposits may often shake up to form perfectly good suspensions for formolisation and dilution to standard opacity, they yield on other occasions suspensions in which many of the bacilli remain more or less agglomerated. The latter suspensions are always undesirable for use in any kind of observations on serum agglutination, and they were put aside as quite unsuitable for experimentation, if, after the usual period of heating in the water-bath, followed by 24 hours' standing at room temperature, there was any deposit whatever at the foot of the control tube, or more than a faint granulation to be seen (with a lens) in the fluid.

B. typhosus.

An actively motile agglutinable bouillon culture of *B. typhosus* (T.e.)* was taken from a strain then in use, and a stock of formolised agglutinable culture of standardised opacity was prepared from it. A subculture was preserved in agar stab, and a subculture was made in the agglutinating serum of rabbits immunised with T.e., diluted 1 to 9 bouillon. The latter culture grew in the form of flocculi of agglutinated bacilli, which sank to the foot of the tube. It was carried on at intervals of 48 hours in small tubes in a succession of cultures in the diluted serum through nine passages. The serum dilutions were tested for sterility before use by incubation for 48 hours at 37° C.

The successive subcultures were always made from the top of the fluid, care being taken not to disturb the deposit. In the first and second cultures this fluid looked clear; but, in the later cultures, an increasing turbidity of the fluid appeared, in addition to the flocculent deposit at the foot of the tube. This appearance suggested that an increasing number of the bacilli present were insusceptible to the agglutinating action of the serum bouillon medium. Nevertheless, the microscope often showed the presence of small clumps among the free bacilli.

From the fifth and ninth serum bouillon culture ordinary bouillon cultures were made, and, from these, subcultures (T.e. 5 and T.e. 9) were prepared

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* The same strain as that denoted T (E) in the paper by Gardner and myself already referred to (4).

24 hours later in 250-c.c. flasks containing 100 c.c. of bouillon. The latter were grown for 24 hours at 37° C., and then well shaken up and formolised. They were placed in cold storage and shaken up several times a day for 3 or 4 days, and were subsequently diluted with formolised normal saline solution to standard opacity. Microscopic examination before formolisation showed that T.e. 5 was almost entirely in the non-motile phase, T.e. 9 short and very highly motile. Serums were prepared by inoculating rabbits intravenously with 1 c.c. of these formolised cultures, and taking blood on the eighth day. Rabbit 1 received 1 c.c. of culture T.e. and serum was prepared (serum I, 1); a month later it received 1 c.c. of culture, T.e. 5, yielding serum I, 2, and, after the lapse of a month more, it received 1 c.c. of T.e. 9 and yielded serum I, 3.

On the same day as the last of these inoculations, rabbits 2 and 3 received intravenous inoculations of 1 c.c. of cultures T.e. 5 and T.e. 9 respectively, and serums II and III were subsequently obtained from them by bleeding on the usual day (eighth).

These serums, together with a 100-unit standard serum (Dreyer), were then tested out against the cultures T.e., T.e. 5 and T.e. 9, using the series of dilutions 1 in 25, 50, 125, 250, etc., of Dreyer's series, and recording the results in Dreyer's notation of total (t), standard (s), trace (tr), and intermediate values (*e.g.*, s−, tr+) as required. The tubes were read after 2 hours in the water-bath at 54° to 56° C., followed by 15 minutes' standing at room temperature, and again after 24 hours at room temperature.

Table I.—Showing Dilutions of Serums yielding "Trace" Agglutination.

Culture.	2 hours' readings.				24 hours' readings.			
	T. 36	T.e.	T.e. 5.	T.e. 9.	T. 36.	T.e.	T.e. 5.	T.e. 9.
100-unit Standard serum...	750	800	10	1,200	1,200	1,000	900	2,500
Serum I, 1	—	1,600	20	2,500	—	1,900	800	4,000
Serum I, 2	—	5,000	450	4,500	—	6,000	1,900	5,500
Serum I, 3	—	4,500	450	4,500	—	6,000	1,900	9,000
Serum II	900	1,900	100	2,200	1,300	2,200	2,000	2,600
Serum III	18,000	10,000	20	18,000	20,000	20,000	175	45,000

Serum I, 1 from Rabbit 1, inoculated with 1 c.c. T.e.
„ I, 2 „ 1, „ „ 1 c.c. T.e. 5, a month later.
„ I, 3 „ 1, „ „ 1 c.c. T.e. 9, a month later still.
„ II „ 2, „ „ 1 c.c. T.e. 5.
„ III „ 3, „ „ 1 c.c. T.e. 9.

The complete record of these tests is omitted from considerations of space. But the end-point readings, after 2 hours and after 24 hours, are presented numerically in the subjoined Table (Table I), where the figure given represents the dilution of the serum observed for a reading of trace (tr), or estimated from an observed end-point reading of trace plus (tr+) or trace minus (tr−).

From this Table, and in connection with it, the following points emerge:—

1. In the culture T.e. 5 the bacillus has become distinctly dys-agglutinable. The 2 hours' readings show a very low agglutination titre even in the three serums prepared with this culture itself, either alone (serum II) or following a previous inoculation of T.e. (serums I, 2 and I, 3).

In the 24 hours' readings the end-point titre has advanced to a fair height, except in the case of serum III. But it is to be stated in this connection that, in the tests made on this culture (T.e. 5), *no tube in any series showed a reading higher than trace plus* (tr+), even when as many as six or seven tubes in series all showed some agglutination, save in the case of the two serums I, 2 and I, 3, where, after two and three inoculations respectively, the serum of rabbit 1 gave marked agglutination (total or standard) in the first two dilutions (1 in 25 and 1 in 50) after 24 hours. Even in these cases the agglutination was of the "dysenteric" type, described by Gardner and myself as characteristic of so-called "inagglutinable" strains of *B. typhosus.* That is to say, the clumps were small, compact, and slowly formed, in contrast with the rapidly formed, large and fluffy flocculi of ordinary typhoid agglutination.

2. The culture T.e. 9, obtained by sampling the supernatant fluid of the ninth successive subculture in serum bouillon, is obviously not at all dys-agglutinable. On the contrary, it is much more agglutinable than the original T.e. It would seem to follow that, at this stage at any rate, the change produced by growth in diluted agglutinating serum *cannot be one of progressive diminution of agglutinability in the whole population.*

It is rather a mechanical separation (by clumping and sedimentation) of the more agglutinable individuals from the less agglutinable, probably accompanied by facilitation of the growth and multiplication of the latter, particularly in so far as they remain unclumped.

But since, as already mentioned, the supernatant fluid often contains (microscopically) many small undeposited clumps, it might easily happen, and presumably did happen in the case of culture T.e. 9, that the sample taken for subcultivation in ordinary bouillon contained enough (clumped) motile agglutinable bacilli rapidly to outgrow the dys-agglutinable non-motile individuals in the course of two successive subcultures in bouillon, the second of which constituted culture T.e. 9. This point is further illustrated in a later experiment.

So far there had been obtained from the culture T.e. cultures T.e. 5 and T.e. 9, the former definitely dys-agglutinable, the latter a good deal more agglutinable than T.e., in fact, two and a half times as agglutinable when referred to standard serum.

3. Culture T.e. 9 produced in rabbit 3 a serum of very high agglutinating power for T.e. and T.e. 9. This, however, does not indicate that in T.e. 9 one has isolated a specially powerful antigenic form of the bacillus. It only shows that rabbit 3 was a particularly good subject for the production of agglutinins. For on looking at the serums obtained from rabbit 1, it is seen that T.e. 9, which was used for the third inoculation, did not in this animal cause any increase of T.e. agglutination above its previous level.

On the other hand, it is very evident that T.e. 9 was remarkably poor in antigenic power for the dys-agglutinable bacillus T.e. 5. For whereas serum III (T.e. 9) was nine or ten times as powerful (24 hours readings) in its action on T.e. 9 as serum I, 1 (T.e.), it was only about one-fifth as strong against T.e. 5. That is to say that in culture T.e. 9 the antigenic power of the original T.e. has been *reduced* to about one-fiftieth as concerns T.e. 5.

4. Serum II (T.e. 5) is seen to be much more powerful in its action on T.e. 5 culture than either of the other serums from single inoculations, and as powerful (24 hours readings) as the serums I, 2 and I, 3, though these serums act two or three times as strongly against cultures T.e. and T.e. 9 as does serum II. Nevertheless, T.e. 5, which has become markedly dys-agglutinable, still retains at this stage good antigenic power in forming agglutinins for T.e.

There is, however, some evidence that its antigenic power for T.e. has undergone reduction, since though serum II (T.e. 5) is definitely stronger than serum I, 1 (T.e.) for the original culture T.e. (say 16 per cent.), it was perceptibly weaker in its action on culture T.e. 9 (say 7 per cent.).

In view of the foregoing considerations, it would seem that by growing the bacillus T.e. in agglutinating serum one was in process of separating out, whether by the mechanical action of clamping and sedimentation alone, or by this combined with an influence favourable to the preferential multiplication of the less agglutinable members of the population, a dys-agglutinable form of the bacillus; and that incidentally one also isolated a particularly highly agglutinable form, T.e. 9.

In this latter culture dys-agglutinable elements were much less well represented than in the original T.e. from which both had been derived, as shown by its feeble antigenic action in relation to culture T.e. 5, when used for the production of agglutinating serum. But that they were by no means absent is rendered probable by an interesting observation which now requires mention, namely, that in the numerous agglutination tests made with T.e. 9 culture a faint haze of unagglutinated bacilli always remained in the supernatant fluid of tubes that would otherwise have been recorded as exhibiting "total" agglutination owing to the complete deposition of the large fluffy flocculi of agglutinated bacilli.

It is of interest to note that, even at this comparatively early stage of differentiation both serum II (T.e. 5) and serum III (T.e. 9) act more strongly on their own homologous culture than upon the standard culture T. 36, though this derived itself (at a much earlier date) from the same original source as culture T.e.

Further investigation of the facts observed was attempted by making a similar experiment in duplicate (in 1 to 3 dilution of serum bouillon) and endeavouring to lead cultures of the bacillus along two diverging paths by making each subculture in one series of tubes from the top of the fluid in the preceding culture, and in the other series making each subculture from the deposit of agglutinated bacilli at the foot of the preceding tube.

The experiment was carried on in both directions through nine successive subcultures (18 days) in serum bouillon. Cultures in ordinary bouillon were made from both series at each stage and formolised after 24 hours' incubation, in the hope of obtaining in the one series an increasingly dys-agglutinable culture, and in the other one increasingly hyper-agglutinable.

But the experiment failed in this respect, owing to the fact (which it revealed) that not only did the supernatant fluid of these serum-bouillon cultures often contain a good many microscopic clumps of agglutinated bacilli, but also the deposit held many unagglutinated (dys-agglutinable) bacilli in the interstices of the flocculent mass. Thus the results yielded cultures no more advanced in the desired directions respectively than T.e. 5 and T.e. 9.

The question of how far the effect of growth in agglutinating serum might consist in the mere mechanical separation of pre-existing agglutinable and dys-agglutinable elements in a given culture was next considered. It was found that it is *sometimes possible* to obtain a dys-agglutinable non-motile culture of *B. typhosus* at the first attempt by simply putting up, with due precautions, a 1-in-10 agglutination test with living culture T.e. in a plugged sterile centrifuge tube, incubating at 37° C. until agglutination was well advanced, and then centrifugalising down the agglutinated bacteria, and making a culture or plating from the supernatant fluid.

From this it follows that, at any rate in a considerable number of cases, there must be numerous dys-agglutinable individuals present in an ordinary bouillon culture of *B. typhosus*.

Moreover, when this method fails to give a dys-agglutinable bacillus, as may occur either because dys-agglutinable individuals are relatively few in number in the original culture (or possibly absent), or because they rapidly revert on cultivation in bouillon, success may often be obtained by combining the method of successive cultures in serum-bouillon with severe centrifugalisation of each culture to deposit all clumps before proceeding to the next subcultivation.

In another experiment, the bacillus T.e. was grown in media containing agglutinating serum for a period of four months. The successive subcultures were made at first every second day, then once a week, and later still at intervals of two or three weeks. At the end of this period a subculture was made in ordinary bouillon. On microscopical examination, after 24 hours' incubation the bacilli were quite non-motile. Many fields were examined and no motile individual seen. From this bouillon culture (A) platings were made on a set of agar plates (*vide infra*), and a flask containing a litre of bouillon was inoculated and incubated for 24 hours. The resulting growth (T. dys.) consisted entirely of non-motile bacilli so far as could be seen, and after formolisation and dilution to standard opacity it was found to be very highly dys-agglutinable. It showed characters similar to those described by Gardner and myself (*loc. cit.* (4)) in our so-called "inagglutinable" cultures, and gave *no agglutination* whatever with 100-unit standard serum even in 1 in 25 dilution after 2 hours in the water-bath.

It also *failed to absorb* any appreciable quantity of agglutinins from standard serum. Thus two portions of a particular standard typhoid serum were taken and diluted 1 in 10 the one (*a*) with standardised agglutinable culture T. 36, the other (*b*) with the formolised culture T. dys. The tubes were kept in the water-bath at 54°–56° C. for 4 hours, and subsequently at the room temperature for 24 hours. In tube (*a*), T. 36 was totally agglutinated when first examined at the end of 2 hours. In tube (*b*), T. dys. showed no agglutination after 2 hours, but a trace (tr) at the end of the 4 hours in the water-bath, and 24 hours later it showed a weak standard (s —) agglutination.

Both tubes were then centrifugalised, and the supernatant fluid removed and denoted serum (*a*) and serum (*b*) respectively, and tested along with the original standard serum on culture T. 36 with the results shown in detail in Table II.

The sample of serum absorbed with T. 36 is seen to have been reduced to something between one-fourth and one-fifth of its original agglutinating power for T. 36; whereas that absorbed with T. dys. shows no measurable loss at all of agglutinins for T. 36, but on the contrary the appearance of a *slight increase*. That is to say T. dys. was not only highly dys-agglutinable, but it was also incapable of absorbing appreciably ordinary typhoid agglutinins.

Table II.

Culture T. 36.	Readings at:	25	50	125	250	500	1000	2500	5000	Controls.
Standard serum	2 hours	t	t	t	t—	s—	tr	0	0	0
	24 „	t	t	t	t—	s+	tr	0	0	0
Serum (*a*)	2 „	t	s+	tr	tr—	0	0	0	0	0
	24 „	t	t	tr+	tr—	0	0	0	0	0
Serum (*b*)	2 „	t	t	t	t—	s	tr	0	0	0
	24 „	t	t	t	t	t—	s—	0	0	0

Standard serum 100-unit standardised serum (Dreyer).
Serum (*a*)—Standard serum absorbed with T. 36.
Serum (*b*)— „ „ „ T. dys.

It is important to add that T. dys. was subsequently brought back to the eu-agglutinating phase. After a long residence in bouillon with numerous subcultures its lineal descendants were found to have reverted to the motile phase, and gave good agglutination with standard typhoid serum. A very similar result was subsequently obtained with a highly dys-agglutinable culture of *B. paratyphosus B.*

It is clear that observations of this character must profoundly modify our views regarding the meaning and importance of serological differences, and the results obtained by absorption methods, particularly when such differences are only quantitative and not qualitative. They suggest that "serological strains" of bacteria, even when apparently permanent, may represent no more than particular phases of activity of the bacterial type concerned. As to how such phasic differences may arise we have at present little evidence. How they are maintained for long periods through successive generations is a problem that urgently demands investigation. But it must certainly be admitted as conceivable that changes which can be induced in test-tube experiments, may on occasion also be produced in the body of an infected individual. *If growth in the presence of agglutinating serum under laboratory conditions can lead to serological changes in one or other direction, it must be accepted as possible that in the living body the agglutinin-containing fluids and agglutinin-producing tissues of the animal may also under suitable conditions exert similar influences.* In this connection the occasional isolation of "inagglutinable" typhoid bacilli from cases of typhoid fever, and the serological diversity of the strains of dysentery (Flexner) isolated during recent epidemics of dysentery afford suggestive evidence.

The bouillon culture (A) of which T. dys. was an immediate subculture had been plated out on agar as already mentioned. From these plates 20 colonies were picked off into bouillon on each of the succeeding 3 days. They were incubated for 24 hours, and then examined for motility and formolised for agglutination tests. All these 60 cultures were highly dys-agglutinable, and they were all non-motile, with the exception of one out of the third set of 20. This culture which, it will be noted, had grown for 5 days on serum-free media and had been three times subcultivated (once on agar and twice in bouillon), *had begun to revert*, and showed a certain proportion (possibly as high as 20 per cent.) of motile individuals among the mass of non-motile elements. It is interesting to observe that this proportion of motile elements was not sufficient to render the culture eu-agglutinable.

So far, then, as the evidence from the bouillon subcultures of sixty colonies goes, the original T. dys. culture consisted entirely of non-motile bacilli.

It is not necessary on the present occasion to go into much detail in describing results which showed *increase of agglutinability and the occurrence of hyper-agglutinable forms.*

The culture T.e. 9 exhibited a degree of hyper-agglutinability. It was about two and a half times as agglutinable by standard serum as T.e. or T. 36. Furthermore, the culture T. 36, with which it was compared, was itself a highly agglutinable culture. It had an index of 7, that is to say, it was nearly three times as agglutinable as the original standard agglutinable culture on which Dreyer's unit was chosen, whose index was 2·5. If, as I suggest, this original standard be taken as the mean standard of eu-agglutinability, the culture T.e. 9 was seven times as agglutinable as that standard, and may be regarded as distinctly hyper-agglutinable. In further experiments, a still more highly hyper-agglutinable culture (about twice as agglutinable as T.e. 9) was readily obtained by growing the bacillus in serum bouillon, centrifugalising out the flocculent clumps at an early stage, and making the succeeding serum bouillon culture from the deposit, and so on, instead of working from the supernatant fluid, as in the pursuit of dys-agglutinable forms.

Similarly, starting from a dys-agglutinable culture, one can frequently restore it to eu-agglutinability by the same procedure. Needless to say, all these operations demand rigorous precautions against accidental contamination of the cultures. The method has a practical application which deserves mention.

Strains are sometimes met with in the case of *B. typhosus* (and the same is true of a number of other bacteria), which are "bad agglutinators." Or it may be that a strain, which for a period (possibly for years) has yielded excellent eu-agglutinable cultures, begins, for totally unknown reasons, to give bad agglutinators. Sometimes these " bad " cultures are really more or less dys-agglutinable. They probably contain a fairly large proportion of dys-agglutinable individuals, so that, in a series of agglutination tubes, a haze of opacity remains in those which should show total agglutination, and the series tails off in a succession of " traces " instead of ending sharply. Such a culture cannot be used for standardisation. At other times they are self-agglomerated (self-agglutinated, auto-agglutinated) growths, which may refuse to shake up into uniform suspensions. These difficulties can usually be overcome by repeated daily subculture in bouillon for 14 days or more, as recommended by Dreyer. But that plan does not always succeed. In such cases a eu-agglutinable culture can often be obtained without much difficulty by the methods already described, working from the deposits if the culture was dys-agglutinable, or from the supernatant fluids if it was in a self-agglomerating phase. In confirmation, it may be stated that Dr. A. D. Gardner, to whom I communicated the method, informs me that on one occasion it enabled him to obtain a good agglutinator from an otherwise intractable culture. A similar separation can also *sometimes* be obtained by mere plating and selection of colonies.

B. paratyphosus A., *B. paratyphosus B.*, *B. œrtrycke B.*, *B. enteritidis* (Gaertner), *B. coli*, *B. dysenteriæ* (Shiga), *B. dysenteriæ* (Flexner), V, W, X, Y, Z, *Vib. choleræ.*

All these organisms were tried during the summer and autumn of 1920, at the same time as the earlier experiments with *B. typhosus,* by the method of successive subcultures in diluted agglutinating serums. A dys-agglutinable culture of *B. paratyphosus A.* was readily obtained ; one of *B. paratyphosus B.*, with greater difficulty, owing chiefly to a strong tendency to revert when brought into ordinary bouillon culture. *B. coli* also yielded a dys-agglutinable non-motile phase. *B. œrtrycke* and *B. enteritidis* (Gaertner), were found very difficult to manage.

On one occasion only, in each case, was a non-motile dys-agglutinable bouillon culture obtained in a test-tube. Massive subculture in flask seemed to lead to immediate reversion to the motile eu-agglutinable phase, and some of the cultures were hyper-agglutinable as compared with standard cultures when tested with standard serum. In another attempt, made with the same strain of *B. enteritidis* (Gaertner), during 6 weeks of this year (1921), though non-motile or largely non-motile colonies were several times obtained on agar plates from serum bouillon cultures, transference to bouillon invariably led to immediate reversion.

Vib. choleræ.—No dys-agglutinable phase has been obtained at present in this vibrio.

All the foregoing organisms show a tendency to the *formation of a pellicle* in serum-bouillon culture. *B. typhosus*, occasionally only and very delicate; *B. paratyphosus* A and B, and *B. coli*, fairly often and well-marked; *Vib. choleræ*, frequently; *B. œrtrycke* and *B. enteritidis* (Gaertner), very frequent and very heavy, often appearing to contain as much growth in the pellicle as in the deposit at the foot of the tube after 48 hours. These statements apply to cultures made in narrow agglutination tubes.

B. dysenteriæ (Shiga) yielded a dys-agglutinable culture after eleven passages in serum-bouillon of 1 in 4 dilution.

B. dysenteriæ (Flexner), V, W, X, Y, Z.—These five strains of Flexner dysentery gave interesting results after six passages through 1 in 4 bouillon dilutions of their appropriate serums. Bouillon cultures were made in flasks from the sixth passages, and subsequently formolised and diluted to standard opacity. V, W, and Z cultures were dys-agglutinable; X was not perceptibly altered; and the Y culture possessed markedly increased agglutinability, being four times as agglutinable as the standard culture against which it was tested. Of the three dys-agglutinable cultures, Z gave no agglutination at all in 1 in 25 standard Z serum at the end of 4 hours in the water-bath, and no more than a trace minus at the end of the subsequent 24 hours at room temperature. W gave trace minus (tr —) at 1 in 25 after 4 hours, trace plus (tr +) at 1 in 25 and trace (tr) at 1 in 50, 24 hours later. V, on the other hand, though giving no reading as high as trace plus (tr +) in any dilution, showed traces of agglutination in every tube up to 1 in 1000, thus reaching the same actual end-point with its "traces" as did the corresponding standard V culture, which gave totals up to 1 in 250, standard (s) at 500, and trace minus (tr —) at 1000.

The discussion of the relation of these dys-agglutinable and well-agglutinable forms of bacteria to the R and S forms recently described by Arkwright (*loc. cit.*), and the description of their morphological and other characters, is reserved for a future occasion.

Summary and Conclusion.

1. Evidence has been brought forward that in the enteric and dysenteric groups of bacteria dys-agglutinable and hyper-agglutinable forms or phases occur, and can be produced experimentally by the methods described.

2. Both forms may be obtained from one and the same eu-agglutinable strain of a bacillus, and both may revert, or may be converted mutually the one into the other.

3. In agglutination tests carried out in the ordinary manner, a highly dys-agglutinable bacillus may fail to agglutinate at all (at 1 in 25) with a serum that agglutinates the culture from which it was derived up to 1 in 1000 or more. It may also entirely fail to absorb from the serum any appreciable quantity of the agglutinins specific to that culture.

4. These results appear to necessitate a considerable modification of current theories regarding the value of absorption tests as a means of determining bacterial affinities, but may help to throw some light on the difficult problem of "serological strains." They show how necessary it is to reserve one's judgment, where conclusions are drawn regarding true differences of bacterial type, in cases where the differentiation rests solely on agglutination and absorption tests; since differences of such remarkable degree are shown to exist between different individuals among the population of a single culture.

I desire to express my indebtedness to Dr. A. D. Gardner, who kindly made independent readings of a number of my agglutination tests, and to Miss Edith F. Stubington for frequent and willing help in many of the more laborious experiments.

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